

SODIUM AND CHLORIDE BALANCE IN THE KILLIFISH *FUNDULUS HETEROCLITUS*

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The euryhaline killifish *Fundulus* has been used extensively in physiological research but no overall picture is available of salt balance in this fish in sea water and fresh water. Indeed knowledge of salt balance in fishes generally is still very incomplete. It has been known for half a century that marine teleosts maintain a blood concentration below that of sea water (Bottazzi, 1906). In 1930 Smith demonstrated that marine teleosts maintain water balance by drinking sea water and excreting the salt extra-renally. The availability of radioactive isotopes has provided more detailed information on salt movement in the last 15 years and has helped to show that the rate of turnover of sodium and chloride ions in several species is much greater in sea water than in fresh water (Mullins, 1950; Gordon, 1963; Motais and Maetz, 1964). Mullins attributed the high influx in sea water entirely to the drinking of the medium and Gordon (1963) and House (1963) concurred with this hypothesis, but Motais and Maetz showed that in the flounder *Platichthys*, drinking accounted for only part of the total influx. However, Motais and Maetz measured the drinking rates of only four fish and used Smith's original technique. It is of interest to note that these appear to be the first direct measurements of drinking rates since Smith's original work. In a paper which has appeared since this work was completed Motais, Romeu and Maetz (1966) have shown that the high rate of exchange in sea water teleosts is due in some fish, *e.g.* *Platichthys*, mainly to exchange diffusion while in others, *e.g.* *Fundulus*, the exchange diffusion component is small.

The present work is an attempt to provide a balance sheet for salt uptake and output in a well-known euryhaline fish. Experiments with fish are often complicated because these animals are very sensitive to handling, diuresis and other changes being induced both by mechanical damage and by shock. The present experiments have been devised to reduce handling to a minimum. To this end fish were loaded by placing them in a radioactive solution, not by injection, and drinking experiments were devised in which it was not necessary to ligate the anus.

MATERIALS AND METHODS

The fish were *Fundulus heteroclitus* weighing between 2 and 5 gm. Most of the fish were collected by the Woods Hole Marine Biological Laboratory Supply Department but in some cases, in order to keep handling to a minimum, fish were collected in glass minnow traps set for a few hours in nearby brackish ponds. The

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fluxes measured in these latter fish were not significantly different from those in fish from the laboratory tanks.

Sea-water-adapted fish were kept in running sea water. Experiments were performed in sea water, an artificial fresh water of reproducible composition, and 40% sea water, approximately iso-osmotic with the blood. The sea water was pumped from some distance offshore and therefore varied slightly in salinity and temperature. The sodium content varied between 420 and 435 mM/L., 30.7–31.8‰ salinity, and the temperature between 17° and 20° C. except for a short time when it reached 23° C. The great majority of experiments were carried out at $20 \pm 1^\circ$ C. The assumed sodium and chloride contents of the sea water, used in the calculations which follow, will be 425 mM Na/L. and 495 mM Cl/L. Forty per cent sea water was prepared by dilution of sea water with tap water and "fresh water" was prepared by diluting sea water to 1 mM/Na/L. with distilled water in order to give a medium of known and reproducible composition. Both 40% sea water and fresh water tanks were aerated and kept in trays of running sea water so that their temperature remained within 1° C. of that of the sea water. All animals were adapted for at least three days before experiments. Fish were fed every two or three days on chopped clam.

Total body sodium and potassium were measured by dissolving weighed fish, dried with tissue paper, in a minimal quantity of concentrated nitric acid and measuring the sodium concentration on a Coleman flame photometer. Sea water sodium and potassium were also measured with the flame photometer. The sodium/potassium ratio in sea water is so high that potassium readings are enhanced, and therefore, potassium in sea water was measured using a standard containing a similar sodium/potassium ratio to sea water. Chloride was measured on a Cotlove chloridimeter, total body chloride being estimated after homogenizing fish in distilled water and centrifuging to remove the bulk of the solids.

Measurement of influxes and effluxes

The isotopes were ^{24}Na , ^{36}Cl and ^{82}Br . Chloride 36 is a weak beta-emitter and the sensitivity of the aluminum-covered scintillation crystal to ^{36}Cl is necessarily very low but it was still possible to obtain significant counts from living animals. Much greater sensitivity could have been obtained with liquid scintillation but this would have required the sacrifice of the fish. Bromide 82, a gamma-emitter, was used in a number of experiments as a substitute for chloride.

Fish were loaded by placing them in 250 ml. of aerated medium containing 50 to 250 μc . of ^{24}Na , ^{36}Cl or ^{82}Br . After a suitable period of loading the animals were washed for 5 to 10 minutes and then transferred to a 100- or 250-ml. efflux bath. At suitable intervals of time 5-ml. aliquots were removed from the efflux bath and their activity was measured using a Nuclear-Chicago crystal scintillation counter, after which the aliquots were returned to the bath. At the end of the experiment the activity remaining in the fish was determined. If A_w was the total activity in the efflux bath and A_f the activity in the fish at time T then the rate constant of the efflux, K_E , can be calculated as follows:

$$K_E = \frac{1}{T} \log_e \left(\frac{A_f + A_w}{A_f} \right) \quad (1)$$

In sea water or 40% sea water the washing bath is effectively an infinite pool of salt but in "fresh-water" solutions the salt content of the bath was of similar magnitude to the salt content of the fish. The specific activity of the ions in the bath, therefore, changed during the course of the experiment. In these circumstances the equations for a two-compartment system were applied (Solomon, 1949). If Na_w and Na_f are the sodium contents of the medium and fish, respectively, K_1 the rate constant for the sodium in the medium, *i.e.* that fraction of sodium in the bath that exchanges per unit of time, and K_2 that for the sodium in the fish, then the flux of sodium through the fish, when in sodium balance is:

$$K_1 Na_w = K_2 Na_f$$

The declining activity in the medium after time T is given by

$$A_{wt} = (A_{w0} - A_{w\infty})e^{-(K_1+K_2)T} + A_{w\infty}$$

where A_{w0} is the initial activity in the medium, $A_{w\infty}$ is the activity in the medium at equilibrium and A_{wt} is the activity in the medium at time T . The increasing activity in the fish is given by

$$A_{ft} = A_{f\infty}(1 - e^{-(K_1+K_2)T})$$

where A_{ft} is the activity in the fish at time T and $A_{f\infty}$ is the activity at equilibrium. Similarly, when the loaded fish is placed in an inactive medium

$$A_{ft} = (A_{fa} - A_{f\infty})e^{-(K_1+K_2)T} + A_{f\infty}$$

and

$$A_{wt} = A_{w\infty}(1 - e^{-(K_1+K_2)T})$$

During the short wash between influx and efflux some activity is lost to the washing solution, which was allowed for by extrapolating $\log_e A_{ft}$ back to the time of the beginning of the wash.

Sodium ions in *Fundulus* are distributed amongst several components. The greater part of the sodium, *ca.* 45 mM/kg. fish, behaves as a single component. This is probably constituted by the sodium in the blood and extracellular fluids, together with most of the cellular sodium. This sodium exchanges rapidly with sodium in the medium when the fish are in sea water but slowly when the fish are in fresh water. A small part of the sodium in the fish, *ca.* 3–4 mM/kg. fish, exchanges only slowly with the environment. This sodium may be situated in the bone and possibly to some extent in cells and connective tissue. Sodium in the gut lumen will form a third component in sea-water-adapted fish. Motais and Maetz (1964) found that in the flounder the specific activity of the gut sodium was only half that of the plasma sodium for some time after transfer to active sea water. This gut sodium may account for much of the difference in total body sodium between sea-water- and fresh-water-adapted *Fundulus* (see below). The presence of these various compartments complicates the interpretation of gross influx and efflux rate constants.

The total ion content of an animal may be determined either by chemical analysis or from the specific activity of the loading solution and the total activity

of the animal at equilibrium when

$$\frac{A_{f\infty}}{Na_f} = \frac{A_{w\infty}}{Na_w}$$

If the fish has been loaded for a long period so that the specific activity of all compartments reaches that of the loading solution and is then transferred to a large volume of inactive solution, the declining activity in the animal will be the sum of a number of exponentials. The rate constant of the decline will be highest initially as the blood/extracellular fluid compartment unloads but will become smaller as the slower compartments, in series with the blood, predominate. A logarithmic plot of the activity against time can only be graphically analyzed into a number of exponentials, if the time constants of the exponentials are widely different. If a multicompartmental animal is loaded for a short time only and then transferred to an inactive solution, the blood which exchanges directly with the medium will have the highest specific activity and effects of the slower compartments will be reduced. A rate constant determined in this way will approximate to that of the blood sodium alone. Similarly the determination of influx rate constants will also be affected by the presence of several compartments. If the ion behaves as if it were all in one compartment then the rate constant of the influx, K_1 , may be calculated from the equation

$$K_1 = \frac{1}{T} \log_e \left(\frac{A_{f\infty}}{A_{f\infty} - A_{ft}} \right) \quad (2)$$

However, when there is more than one compartment the blood/extracellular fluid sodium compartment will load more rapidly. If the influx is of short duration the influx rate constant K_1 calculated from equation (1) will represent the sodium influx expressed as a fraction of the total body sodium. However, in longer experiments, equation (2) will give progressively smaller values of K_1 as the inactive sodium remaining in the animal, $A_{fx} - A_{ft}$, is identified more and more with the slowest components.

To summarize: short-term influx experiments will give an effective rate constant for the whole animal while short-term efflux experiments especially if following a short-term load, will give initially a rate constant approximately to that of the fast blood/extracellular fluid compartment alone. This is borne out by the experiments. In sea water and 40% sea water the efflux rate constants calculated from equation (1) are greater than the influx constants calculated from equation (2). In fresh water where the gut sodium is small and where the exchange across the body surface is much slower than the exchanges within the animal, the rate constants are not significantly different.

In many experiments A_x was calculated from the specific activity of the loading solution and the average composition of fish adapted to that salinity. As the individual compositions were rather variable each rate constant calculated in this way was subject to some indeterminate error, but the means should not be effected significantly.

In the initial experiments activity in the fish was determined by dissolving the fish in concentrated nitric acid, diluting to a known volume and measuring the activity in a 5-ml. aliquot. However, it was found that with fish 2-5 gm. in

weight the count for the whole fish, when placed head down in a test tube, always lay between 91% and 108% of the count of the fish when dissolved and made up to 5 cc. and the mean count of the whole fish was $96.5\% \pm 1.2$ ($N = 14$) of that of the solution. In some later experiments the live fish were counted and the activity in the fish calculated from this relationship.

The rapid changes of efflux that follow changes of salinity were investigated by measuring activity released into an inactive solution. Fish adapted to sea water were loaded for 12 hours in 200 ml. of sea water containing 1 mC. $^{24}\text{Na}/\text{L}$. After a two-minute wash in running sea water fish were transferred to 200 ml. of inactive sea water. Five-ml. samples were taken every five minutes, counted and returned to the bath. After a suitable period of time the fish were washed for two minutes in running tap water and transferred to 200 ml. of artificial "fresh water" and the procedure repeated. The fresh water was changed every hour. At the end of the experiment the activity remaining in the whole fish was counted. From the successive losses and the final activity, the initial and the rates of loss were calculated. The variation of the rate of uptake with the external concentration was measured as follows. A solution containing 10, or in some cases 30 mM Na/L. and 500 μC . $^{24}\text{Na}/\text{L}$. was prepared. By dilution a series of solutions were prepared of the same specific activity but containing 0.1, 0.3, 1.0, 3.0 and 10 mM Na/L. A fish was first placed in the most dilute solution for 5 minutes, washed in running tap water for 5 minutes and the activity counted. It was then placed in the next most concentrated solution for 5 minutes and the procedure repeated. As the increment of activity gained by the fish in each solution was greater than the total activity gained in the less concentrated solutions, the activity gained in every solution could be obtained with reasonable accuracy.

For example, after 5 minutes successively in solutions containing 0.1, 0.3, 1, 3 and 10 mM Na/L., a fish counted 822, 3165, 8773, 18,604 and 36,427 cpm. Hence the increments between solutions were 882, 2343, 5608, 9831, 17,823, respectively, and the sodium influxes would be proportional to the increments of the counts.

Handling does not affect the rate of uptake to a significant extent. Fish placed repeatedly in a 1 mM NaCl/L. solution containing ^{24}Na took up sodium at a constant rate.

Drinking experiments

Although Smith's demonstration in 1930 that marine teleosts drank sea water is widely known, his experiments were brief and have practically never been repeated. Smith placed the animal in a medium containing phenol red after ligating the anus and later measured the concentration of phenol red in the gut. The method is open to several obvious objections. If any phenol red is absorbed from the gut the apparent rate of drinking will be reduced. The turbidity of the gut contents limits the accuracy of the method and the ligation of the anus makes the experiment unphysiological. The method is only practicable on fair-sized fish. In an attempt to overcome some of these objections and to develop a method applicable to small fish two methods were tried. In the first inulin was used as a marker for drinking and the whole fish was homogenized after washing to remove surface activity. Even if some of the inulin was absorbed from the gut it would still be assayed by the method adopted unless it had been completely metabolized to CO_2 .

This method may give rather high values for drinking as the result of surface contamination of the fish. In the second method ^{35}S -labeled sulphate was used as a marker and the gut alone was assayed. The experiment was limited to 45 minutes to limit uptake from the gut. Preliminary experiments showed that some activity was found in the body, especially the kidneys, indicating some uptake of sulphate from the gut. After two hours in ^{35}S -labeled sea water the activity in the kidneys amounted to 10% that in the gut. Consequently, the values derived by this method will be rather smaller than the real values. The real volume drunk must lie between those given by the two methods. The discrepancy given by the two methods is larger in fresh water than in sea water. This might be due to the fact that any fresh water drunk will be more extensively and rapidly taken up from the gut, while sea water will be taken up more slowly and less completely.

Three solutions, sea water, 40% sea water and 1 mM/L. NaCl, were made up to contain from 10 to 30 μC . ^{14}C inulin per 100 ml. To the active inulin was added 10 times its weight of inactive carrier but the total concentration of inulin never exceeded 0.04%. Fish were placed in the labeled solution for one hour, removed, washed for 5 minutes, and homogenized in 20 ml. of distilled water and 0.5 ml. sea water. The homogenate was centrifuged at 55,000 RPM for 5 minutes and 0.1 ml. of the supernatant was added to 1 ml. distilled water and 15 ml. of Bray's solution (Bray, 1960). The activity was measured on a Packard Tri-Carb liquid scintillation counter. A standard was prepared by homogenizing an inactive freshly killed fish in 20 ml. distilled water and 0.5 ml. of the original drinking solution. This homogenate was also centrifuged and the activity of 0.1 ml. of the supernatant was measured with the liquid scintillation counter. Surface contamination was corrected by allowing a fish to drink for 5 minutes in the labeled 1 mM NaCl solution, treating as above, and calculating the cpm due to surface contamination by using simultaneous equations. The correction for contamination proved to be only 1% of the original cpm.

Preliminary experiments were carried out in which the fish were placed in active solutions for five minutes, 30 minutes, one hour, two hours and four hours. When allowance was made for the surface contamination the activity was found to increase linearly for the first two hours, after which it showed signs of leveling off. This is interpreted as meaning that swallowed inulin begins to be lost from the anus after about two hours. Measurements were therefore made after one hour of drinking. This obviates the need to ligate the anus.

Sulphate

Sea-water and fresh-water solutions were prepared, each containing 100 μC . ^{35}S -labeled Na_2SO_4 in 250 ml. Fish were placed in a solution for 45 minutes, then removed directly to a washing solution containing MS 222. The anaesthetic was added to reduce the possibility of regurgitation into the pharynx during killing and dissection, which was observed in non-anaesthetized fish. When the fish was immobilized the gut was removed and dropped into 2 ml. of 0.01 N Na_2SO_4 after which it was weighed and then chopped into short pieces about 3 mm. long to allow ^{35}S sulphate to diffuse out. After 6 hours of soaking with frequent shaking the samples were centrifuged and 0.5-ml. aliquots were added to 15 ml. of Bray's solution and counted as before.

RESULTS

Total body sodium, potassium and chloride are given in Table I. It can be seen that while total body potassium remains remarkably constant in the different media, fresh-water fish contain barely half as much sodium and chloride as the sea-water ones.

Total exchangeable sodium

The total exchangeable sodium, calculated from the activity in seven specimens of *Fundulus*, after loading for 24 hours in sea water and washing for 5 minutes in inactive sea water, was 85.3 ± 4.0 mM Na/kg. The average exchangeable sodium determined in this way is slightly greater than the total sodium as measured by flame photometry (Table I) although the excess is only equal to the sum of the standard errors. Some of this activity may be due to the activity in the surface mucus not represented in the photometric analyses, which were made on animals whose surfaces had been dried with tissue paper.

TABLE I

Sodium, potassium and chloride content of Fundulus heteroclitus in various media, mM/Kg.

	Na ⁺	K ⁺	Cl ⁻
Sea water	78.7 ± 3.3 (12)	85.2 ± 3.7 (6)	61.3 ± 1.4 (12)
40% sea water	60.6 ± 1.8 (9)	—	50.8 ± 1.2 (12)
Fresh water	48.5 ± 1.5 (10)	80.9 ± 0.7 (6)	31.3 ± 1.8 (12)

Mean $\pm$ S.E. (No. of determinations).

After loading for 48 hours or more the activity in the fish declines after transfer to inactive sea water as the sum of two exponentials. If the activity in the fish after time T is A_{ft} then

$$A_{ft} = A_1 e^{-k_1 T} + A_2 e^{-k_2 T}$$

In three fish in which effluxes were measured over a period of 10 hours the average value of A_1 was 0.93 and that of A_2 , 0.07. The average rate constant for the fast phase (k_1) was 0.55 h^{-1} and for the slow phase (k_2) was 0.16 h^{-1} . In similar experiments with bromide, A_2 averaged only 0.042 and k_2 , 0.028 h^{-1} . Long-term efflux experiments could not be performed with chloride-36 as the sensitivity of the apparatus to beta particles was so low that the counts soon became insignificant.

Rates of efflux

The rate constants of the efflux of sodium, chloride and bromide were determined in each of the three media. The results are given in Table II. The fluxes of both sodium and chloride in fresh water are less than one-twentieth of the fluxes in sea water. The sodium fluxes in 40%, however, were peculiarly variable. This will be discussed further below, but briefly it appears that in this medium some of the fish were behaving approximately as fresh-water fish while others were

still behaving as sea-water fish. The median rate constant for sodium in 40% sea water was about 0.09 h^{-1} but the average (Table II) has been weighted by a few larger effluxes. It is clear that the rate constants of the chloride fluxes are rather larger than those of the sodium fluxes, as might be expected with a smaller ion. Bromide is not a good indicator for chloride as it moves more slowly than chloride in both sea water and 40% sea water. In fresh water the rate of bromide efflux is apparently slightly faster than that of chloride, although the data are too limited to decide whether this difference is significant.

Influxes

When the fish are in equilibrium the influx should be equal to the efflux. In fresh water and in 40% sea water the computed influx and efflux rate constants were similar (Table II), but in sea water the influx rate constants appear to be significantly smaller than the efflux rate constants. As explained above, the efflux

TABLE II
*Rate constants of sodium, chloride and bromide fluxes through
Fundulus heteroclitus in various media, h^{-1}*

EFFLUX	Na^+	Cl^-	Br^-
Sea water	0.462 ± 0.024 (19)	0.874 ± 0.040 (12)	0.429 ± 0.027 (9)
40% sea water	0.135 ± 0.027 (14)	0.146 ± 0.015 (9)	0.087 ± 0.007 (27)
Fresh water	0.017 ± 0.003 (16)	0.031 ± 0.004 (11)	0.037 ± 0.004 (14)
INFLUX	Means $\pm$ S.E. (No. of determinations).		
Sea water	0.261 ± 0.020 (8)		
40% sea water	0.154 ± 0.043 (7)		
Fresh water	0.0120 ± 0.003 (8)		

rate constant is increased by the predominance of the fastest component during a short load, while the influx rate constant is reduced as the function $A_{fx} - A_{ft}$ in equation (2) approximates to the slowest component. The total flux across the body wall may be calculated from the rate constant of the influx multiplied by the total ion content of the animal. In sea water the influx is $20.5 \pm 1.8 \text{ mM Na/kg./hr.}$; in 40% sea water it is $9.3 \pm 2.6 \text{ mM/kg./hr.}$ and in fresh water only $0.58 \pm 0.02 \text{ mM/kg./hr.}$ The true fluxes are probably rather larger than these for the reasons given above. The flux in sea water is more than 30 times as great as the flux in fresh water.

Rate of drinking

The rate of drinking in sea water as measured by inulin averaged 2.30% body wt./hr., but only 1.54% as measured by sulphate. The real value must lie between these two figures. This is several times as large as reported by Smith (1930) or Motais and Maetz (1964) but their experiments were performed on animals weighing several hundred grams. Drinking continues almost unchanged in 40% sea water although the results in this medium were very variable, ranging

from 0.51% to 5.2%. In fresh water the rate of drinking was much lower but was still significant (Table III).

Entry of ions

Salts may enter the fish either through the gut or directly through the body surface. As a result of his experiments, Mullins (1950) believed that the gut was the major or only port of entry in *Gasterosteus* and consequently House (1963) made a similar assumption in interpreting his work on *Blennius*. It is clear that this is not the case in *Fundulus*. In sea water the rate of swallowing is equivalent to only $9.6 \mu\text{M Na/gm./hr.}$, even if we accept the high result of the inulin experiments: in 40% sea water the rate of swallowing is equivalent to only $3.4 \mu\text{M/gm./hr.}$ It follows that in all media the greater part of the influx takes place directly through the body surface. Motaïs and Maetz (1964) found that this was true also in the

TABLE III

Rates of drinking of medium by Fundulus heteroclitus in various media; % body wt./hr.; 20°C.

	¹⁴ C Inulin	³⁵ S Sulphate
Sea water	2.30 ± 0.27 (10)	1.54 ± 0.21 (14)
40% sea water	2.00 ± 0.43 (14)	
Fresh water	0.83 ± 0.39 (7)	0.14 ± 0.05 (14)

Mean S.E. (No. of determinations).

flounder. The most probable site of this influx is the gills. It is unlikely that there is much influx through the skin, scales or fins, which are poorly vascularized.

Routes of efflux

The rate of efflux of sodium in sea water and 40% sea water was determined in two groups of fish in which the ureters were ligated. In each group the average rate of efflux was higher than in normal animals. In sea water, $K_0 = 0.462 \pm 0.024$ (19) for normal animals and 0.610 ± 0.06 (11) for ligated animals. Similarly in 40% sea water $K_0 = 0.135 \pm 0.027$ (14) and 0.217 ± 0.053 (9), respectively. This implies that the anaesthetic and the shock of the operation combine to increase the permeability of the body wall to an extent sufficient to mask any reduction of efflux caused by the absence of urine.

Rate of adaptation to fresh water and the effect of fresh water on the rate of efflux

The rate of loss of sodium in fresh water was so low compared with that in sea water that the process of adaptation was investigated in greater detail.

On transfer from sea water to fresh water the rate constant of sodium efflux dropped from *ca.* $0.4\text{--}0.6 \text{ h}^{-1}$ to *ca.* $0.02\text{--}0.05 \text{ h}^{-1}$ in about 15 minutes. When transferred back to sea water the efflux remained close to the fresh-water level for about four hours and only returned to the sea-water level after about 18 hours (Fig. 1). This marked asymmetry in the effluxes during transfer from sea water to fresh water and from fresh water to sea water shows that exchange diffusion does not

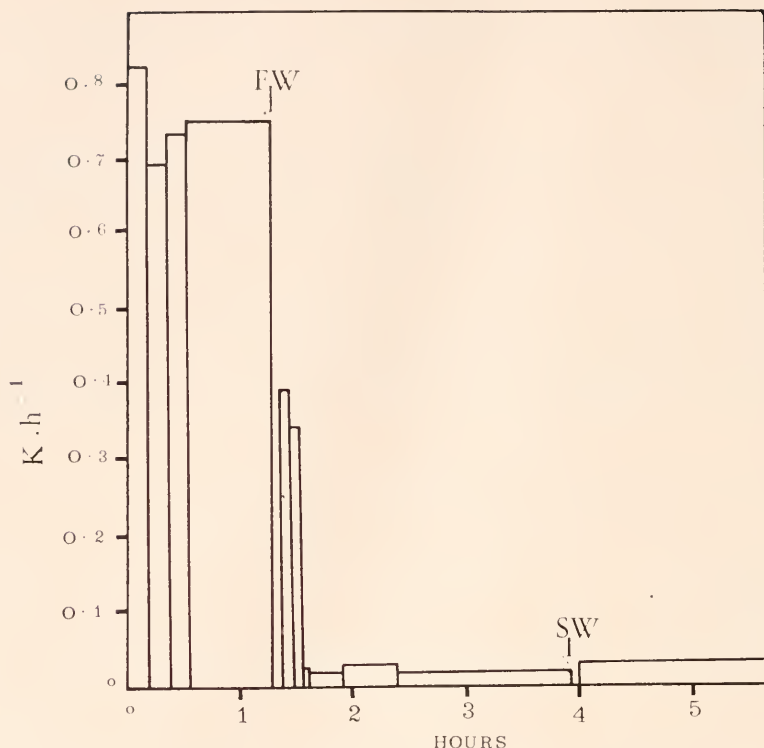


FIGURE 1. Rate constant of sodium efflux in sea water and fresh water. At 1 hour 17 minutes fresh water was run through the bath for a few minutes to remove traces of salt. Similarly at 3 hours 55 minutes sea water was run through the apparatus. At these times no measurements were possible.

contribute substantially to the rate of efflux in sea water. However, it might be argued that the high efflux was due, in the main, to the active extrusion of sodium consequent on the drinking of sea water in order to maintain water balance. After several hours in fresh water the fish might become water-loaded and not start to drink for some time after returning to sea water. To examine this possibility sea-water-adapted fish were transferred to fresh water for limited periods of time, then loaded in active sea water for two hours and the rate of efflux in sea water was then measured during the succeeding two hours. It was found that only 10 minutes in fresh water reduced K_0 to 0.285 ± 0.08 (7) while 15 minutes reduced K_0 to 0.190 ± 0.07 (8).

DISCUSSION

The regulation of the total body salt after transfer between media seems at first sight to be very poor. Black (1948) found an even greater variation in chloride content between sea-water- and fresh-water-adapted *Fundulus* (sea water 55 mM Cl/kg., fresh water 20 mM Cl/kg.) but the low value in fresh water may be related to the very low concentration of chloride (0.07 mM/L.) in her fresh water. Burden (1956) found that the chloride concentration of the blood in sea water

was not significantly different from that in fresh water, while Pickford (1965) has found only a small decline in total osmotic pressure (sea water = 180 mM/Cl/L., fresh water = 162 mM/NaCl/L.) but very recently Motais *et al.* (1966) report that the plasma sodium of *F. heteroclitus* declines from 193 mM/L. in sea water to 156 mM/L. in fresh water. Some part, at least, of the discrepancy between the variable total body sodium and the more constant plasma concentration may be attributed to the high concentration of salt in the gut of sea-water-adapted fish. Motais (1961) and Motais and Maetz (1964) have shown that the sodium in *Platichthys* gut exchanges only slowly with the sodium in the blood.

A large reduction of the sodium and chloride fluxes on adaptation to fresh water has also been found in many teleosts. The sodium flux through *Gasterosteus* was reduced from 11 mM/kg./hr. in a dilute Baltic sea water to 0.7 mM/kg./hr. in fresh water (Mullins, 1950). In *Platichthys* the rate constant of sodium exchange declined from 0.18 to 0.3/hr. in sea water to 0.01/hr. in fresh water (Motais, 1961), while Gordon (1963) showed a similar reduction in chloride fluxes in *Salmo*. However, Mullins (1950) found that in sticklebacks (*Gasterosteus*) adapted to Baltic sea water, containing only 267 mM Cl/kg. water, or 9.5% chlorinity, the rate of uptake of several ions, sodium, potassium, bromide and phosphate, was similar when expressed in the terms

$$\frac{\text{Activity/gm. fish}}{\text{Activity/gm. solution}}$$

Mullins suggested that this result could be accounted for by the assumption that the fish drank the equivalent of 4% of their body weight/hr., but in the light of later work the result must be attributed to a coincidence. Gordon (1963) has measured the rate of efflux of chloride from both normal rainbow trout, *Salmo gairdneri*, and from trout with ligated cloacae. The efflux from trout adapted to sea water was between 10 and 20 times the efflux from trout in fresh water but the variations between the small numbers of individual fish used and between fish at different times of the year makes any conclusions hazardous. Fish with ligated cloacae had an average rate of efflux in sea water about one-third of that of normal animals. Gordon suggested that the renal loss in sea water exceeded the extra-renal loss but this is impossible under the Smith hypothesis of osmoregulation in marine teleosts. The urine of marine teleosts is approximately isosmotic with the blood and contains a high concentration of divalent ions. Holmes (1963) points out that for every liter of sea water drunk the corresponding urine flow must be less than one liter and the sodium chloride loss in the urine can only be equivalent to a small part of the total taken in by drinking. Motais *et al.* (1966) have demonstrated that a rapid decline in efflux takes place in a variety of euryhaline teleosts on transfer from sea water to fresh water. In *Platichthys* a large part of the exchange in sea water is due to exchange diffusion but in *Fundulus* the exchange diffusion component is small, *ca.* 15%. The exchange diffusion component causes an instantaneous reduction in the efflux on transfer to fresh water but in both species a reduction takes place in the permeability of the fish to ions in the hours following transfer which causes a further reduction in efflux.

Practically no direct measurements of drinking rates have been published since Smith's original measurements. Smith estimated the rate of drinking from the

concentration of dye in the gut of fish, with ligated cloacas, kept in sea water containing phenol red. He estimated that eels, *Anguilla*, and sculpins, *Myoxocephalus*, drank between 50 and 200 ml./kg./day, average 60 ml./kg./day, of which three-quarters was absorbed from the gut. The residual fluid was iso-osmotic with the blood and contained mainly divalent ions. Smith suggested that the salt absorbed is excreted extra-renally as a hyper-osmotic solution but this would entail further water loss. It is likely that the ions are secreted directly into the sea water. Keys (1931) and Schlieper (1933) showed that salt was secreted from the anterior end of the body, and it is probable that the gills are the site of secretions although the identity of the cells responsible has been the subject of a long controversy.

Motais and Maetz (1964), using Smith's original technique, estimated that *Platichthys* drank 0.73–1.37% body wt./hr. (mean 1.0% hr.), equivalent to an influx of 5 mM Na/kg./hr. The total sodium flux averaged between 10 and 12.5 mM/kg./hr. Here also the greater part of the influx took place outside the gut. Drinking evidently continues in iso-osmotic sea water, and at a reduced rate, even in fresh water. Frank and Allee (1950), using colloidal thorium dioxide as a marker, also found that *Fundulus diaphanus* continued to drink in fresh water although Bergeron (1956), using colloidal carbon, could detect no drinking in fresh water in *F. heteroclitus*. However, Bergeron was able to detect drinking in solutions as dilute as $\Delta 0.04^\circ \text{C.} \equiv 10 \text{ mM NaCl/L.}$ Neither Bergeron nor Frank and Allee made any quantitative estimates of drinking. In sea water the total influx of sodium amounts to 20.5 mM/kg./hr. Less than one-half of this enters by the gut, while the rest, 11 mM/kg./hr., must enter through the body surface, especially the gills. Until the chloride fractions have been analyzed in detail it is not possible to define the chlorine fluxes exactly but from Table II it seems likely that they too are similar but somewhat greater than the sodium fluxes. A drinking rate of 2.3% per hr. corresponds to an influx through the gut of 11.3 mM Cl/kg./hr. while the total chloride influx may be of the order of 40 mM/kg./hr. It is probable that the passive chloride influx is proportionately greater than that of sodium as the sea-water chloride concentration is higher and the chlorine ion smaller than the sodium ion.

In 40% sea water the rate of drinking as measured by inulin corresponds to a sodium influx of 3.4 mM/kg./hr., again less than half the total. The passive influx must be about 5 mM/kg./hr., suggesting that the permeability to sodium in this medium is similar to that in sea water. In fresh water the passive influx is almost negligible and nearly all the influx must be due to active uptake. This varies with the external concentration in a manner similar to that found in the fresh-water Crustacea (Shaw, 1959, 1961; Shaw and Sutcliffe, 1961). The relationship between active influx, f , and external concentration, M , may be represented by the equation

$$f = f_{\max} \left(\frac{M}{M + C} \right)$$

This equation is similar in form to the Michaelis-Menton equation relating the rate of an enzymatic reaction to the concentration of substrate. In the influx equation, C is the concentration at which $f = \frac{1}{2}f_{\max}$ and is a measure of the affinity of the carrier molecule to the ion. In general the lower the value of C , the lower the

concentration in which the animal can survive. In *Fundulus*, C is about 2 mM Na/L. In fresh-water Crustacea values of C are found as low as 0.2 (*Astacus*) or 0.1 mM/L. (*Potamon*). On this evidence *Fundulus* is not as well adapted to fresh water as these crustaceans and indeed *Fundulus* does not survive well in tap water.

Sodium loss may occur in several ways: by diffusion through the body wall, in the urine and from the gut. In fresh-water fish the rate of urine production is usually less than 1% per hr. and the urine is hypo-osmotic to the blood, so that the renal loss of sodium is small. Meier and Fleming (1962) have shown by ligation experiments that in the plains killifish, *F. kansae*, 28% of the total sodium loss takes place through the kidneys, 10% through the gut and the rest through the body surface. Stanley and Fleming (1964) found that in fresh water *F. kansae* produced 8.3 ml./kg./hr., containing an average of 12.8 mM Na/L. The sodium loss would amount to 0.01 mM/kg./hr.

If we assume a similar distribution of losses in *F. heteroclitus* the sodium loss through the body surface in fresh water will be reduced to ca. 0.36 mM/kg./hr. The chloride flux across the body wall may be independent of the sodium flux when the animal is in equilibrium.

The losses in sea water are more difficult to evaluate since a small passive efflux of sodium will occur even in this medium. The inward diffusion in sea water is of the order of 11 mM/kg./hr. The ratio, passive efflux/passive influx, will be equal to blood concentration/sea water concentration in the absence of any potential difference. The passive efflux in sea water would therefore be about 4 mM/kg./hr. if there were no potential difference. Renal loss in sea-water-adapted fish must be small. Urine flows in marine teleosts are of the order of 1–2% body weight/day (Black, 1957; Holmes, 1963). In the related species *F. kansae*, adapted to sea water, the rate of urine production was 0.87 ml./kg./hr., containing 140 mM Na/L. (Stanley and Fleming, 1964). The renal sodium loss in sea water was therefore 0.12 mM/kg./hr.

Some loss of sodium will take place from the anus. In *Lophius* the sodium concentration of the fluid in the lower end of the intestine was only 56 mM/kg. water (Smith, 1930). The volume of fluid lost from the gut is unknown but can only be a small fraction of the sea water drunk. Again in *Lophius* the sulphate concentration at the lower end of the gut was 6 times as great as in the sea water (Smith, 1930), since sulphate is absorbed slowly if at all; thus at least five-sixths of the sea water taken in had been absorbed. It may be concluded that although some sodium will be lost from the rectum it is unlikely to exceed 10% of that taken in through the gut and is likely to be even less. Sodium loss from the rectum in sea water may therefore be estimated at less than 1 mM/kg./hr. The remaining sodium loss, ca. 16 mM/kg./hr., must be due to the active extrusion of sodium against the concentration gradient. Fleming, Scheffel and Linton (1962) have shown that the active extrusion of sodium chloride by marine teleosts is associated with cholinesterase activity in the gill. The efflux of sodium from *F. kansae* is reduced by 75% by treatment with 5×10^{-6} M eserine.

Influxes, effluxes and drinking rates are all more variable in 40% sea water than in either sea water or fresh water. Some efflux rate constants in fish adapted to 40% sea water are similar to those of fresh-water fish and others to those of

sea-water fish. From this it seems probable that some fish are behaving as sea-water-adapted fish, while others appear closer to fresh-water-adapted fish.

The most striking difference between the sea-water-adapted fish and the fresh-water-adapted fish lies in the permeability of the body wall to ions. It is clear that the most important single change during adaptation to fresh water is the reduction of permeability. An external sodium concentration of 425 mM/L. generates an influx of 11 mM/L. through the body surface. In fresh water a blood concentration of ca. 150 mM Na/L. would generate an efflux of ca. 4 mM Na/L. if the permeability remained unchanged and there was no potential across the body wall, but the observed efflux is about one-tenth of this. If this reduction in permeability did not occur, survival in fresh water would only be possible if the rate of active uptake were increased many times. As was shown in an earlier paper, hypophysectomized *Fundulus* in fresh water fail to survive, in the absence of prolactin, because their permeability does not remain high. Normal fish which have been adapted to fresh water or even placed in fresh water for a short period, maintain a low permeability for many hours after return to sea water (Fig. 1). The rate of turnover of sodium is greatly reduced between two and four hours later after only 15 minutes in fresh water. The rapid, but not instantaneous, adaptation and slow reversal suggest that the permeability is controlled by a hormone released in response to fresh water and/or a falling blood concentration. The exchange diffusion component in the sea water flux is small.

While the advantages of a low permeability to ions in fresh water are obvious, the advantages of a high permeability in sea water are not. Marine fish must work to maintain osmotic equilibrium in sea water and reduction in permeability would reduce this work. The increased permeability observed in sea-water-adapted fish presumably confers some advantage but no convincing suggestion can be offered.

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SUMMARY

Measurements have been made of the rates of influx and efflux of sodium ions in sea water, 40% sea water and fresh water in the killifish, *Fundulus heteroclitus*. Measurements have also been made of the rates of efflux of chloride and bromide ions in the same media. The rate of drinking has been measured using inulin-labeled sea water. In sea water the sodium influx averages 20 mM Na/kg./hr., of which more than half enters by diffusion, the rest by drinking. In fresh water the rate of influx is about 0.6 mM Na/kg./hr., practically all of which takes place by active transport. Adaptation to fresh water is accompanied by a great reduction in permeability to sodium and chloride ions. This fall in permeability takes place within a few minutes of transfer to fresh water but the increase of permeability on return to sea water takes many hours. The relationship between active uptake and external concentrations at low concentrations resembles that found in fresh-water crustaceans.

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